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Docosahexaenoic Acid Reduces Amyloid-β_{1-42} Secretion in Human AβPP-Transfected CHO-Cells by Mechanisms Other than Inflammation Related to PGE_2

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Abstract. The effect of supplementation with the omega 3 polyunsaturated fatty acid (n-3 PUFA) docosahexaenoic acid (DHA) on membrane composition and amyloid-β_{1-42} (Aβ_{42}) secretion was studied in human amyloid-β protein precursor-transfected Chinese Hamster Ovary (CHO) cells. Twenty-four hour incubation with a range of DHA concentrations resulted in a dose-dependent increase in membrane DHA and eicosapentaenoic acid content and a decrease in arachidonic acid content. In addition, DHA supplementation caused a dose-dependent reduction in the secreted Aβ_{42} levels and resulted in a 4–8 fold decrease in extracellular prostaglandin E_2 (PGE_2) levels. Tocopherol, which was added to DHA to prevent oxidation, may have contributed to the effect of DHA, since it slightly decreased extracellular Aβ_{42} and PGE_2 levels when given alone. The addition of selective COX-2 inhibitors Celebrex and curcumin to the culture medium resulted in a significant and comparable inhibition of PGE_2 release, but did not inhibit Aβ_{42} secretion, and even significantly increased Aβ_{42} production in this cell system. Together, the present data show that, whereas both DHA and COX-2 inhibitors may reduce PGE_2 production, only DHA in the presence of tocopherol significantly reduced Aβ_{42} production and concurrently changed membrane lipid composition in CHO cells. It is concluded that in this *in vitro* setting DHA reduced Aβ_{42} secretion through membrane-related, but not PGE_2-related mechanisms.

Keywords: Alzheimer's disease, amyloid-β, COX inhibitor, curcumin, DHA, inflammation, membrane, nutrition

INTRODUCTION

With the aging population, the number of people developing Alzheimer's disease (AD) will increase dramatically. Epidemiological studies have shown that the risk of developing AD decreases with increased intake of fatty fish [1]. Especially the intake of omega-3 (n-3) polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), is associated with this decreased risk. Today, dietary intake of n-3 PUFAs in Western countries is lower than recommended. Especially during the last century, the intake of omega-6 (n-6) PUFAs has in-

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creased at the cost of n-3 PUFAs resulting in a reduced n-3/n-6 ratio [2–4]. Since, both n-6 and n-3 PUFAs compete for the enzyme cyclooxygenase (COX) [5,6], increased intake of n-6 PUFAs leads to a higher production of the n-6 derived pro-inflammatory eicosanoid prostaglandin E_2 (PGE_2).

A key factor in the development of AD is the formation of dense plaques in the patient's brain. One of the components of these plaques is amyloid-β (Aβ), the predominant species being $A\beta_{1-42}$ ($A\beta_{42}$), which is formed by cleavage of the amyloid-β protein precursor (AβPP) by β- and γ-secretases. Interestingly, several studies have shown that PGE_2 itself or stimulation of COX-2 activity leading to increased PGE_2 production, can promote Aβ formation, likely through stimulation of γ-secretase activity [7,8]. Reducing the formation of PGE_2 by increasing n-3 PUFAs [9], might therefore, offer an effective method to diminish the secretion of Aβ.

Studies in transgenic models for AD suggest that Aβ burden, inflammation, and behavioral deficits can be attenuated by pharmacological COX inhibition by non-steroidal anti-inflammatory drugs (NSAIDs) [10–12]. Increased COX-2 activity in AβPP$_{swe}$-PS1$_{dE9}$ mice by transfection with human COX, however, resulted in cognitive deficits that could be reversed by pharmacological COX-2 inhibition, but did not increase Aβ burden [13]. The absence of an effect of COX overexpression on Aβ production *in vivo* may depend on the age of the animals, since it confirmed a previous study where effects were found in 24 months (mo) old, but not in 8–12 mo old double transgenic mice [7] modeling an earlier stage of AD. These results may suggest that existing Aβ deposits may be necessary to elicit COX-2 mediated potentiation of Aβ deposition. This is supported by the fact that elevated levels of PGE_2 and overexpression of COX-2 have been observed in the brains of AD patients [14–16], and that the extent of COX-2 expression correlates with the amount of Aβ and the degree of progression of AD pathogenesis [17].

The present study aimed to test whether *in vitro* supplementation with the n-3 PUFA DHA can lower $A\beta_{42}$ secretion by reducing PGE_2 production. To this end, human AβPP transfected Chinese Hamster Ovary (CHO) cells were supplemented with a range of DHA concentrations, and both amyloidogenic $A\beta_{42}$ secretion and PGE_2 production were measured. The relationship between PGE_2 production and Aβ secretion was further investigated by applying selective COX-1 & -2 inhibitors to the cells. Finally, since DHA supplementation is known to change membrane composition [18,19], and AβPP processing is hypothesized to be influenced by membrane composition [20,21], we also measured membrane phospholipids and membrane fatty acids in the present study.

MATERIALS AND METHODS

Cell culture

CHO-7PA2 cells from a Chinese Hamster Ovary cell line, stably transfected with a cDNA for the 751 form of AβPP bearing the V717F familial AD mutation, and driven by a CMV promoter, were kindly provided by Dr. Selkoe and described in detail elsewhere [22]. Cells were cultured in DMEM/F-12 containing 10% FCS, 2 mM glutamine and 100 μg/ml geneticin (G418) (all components were purchased from Invitrogen Life Technologies, Merelbeke, Belgium). The addition of 10% FCS to the culture medium was necessary to allow DHA supplementation and therefore used in all experimental conditions.

DHA supplementation

For measurement of $A\beta_{42}$, PGE_2 and fatty acid analyses, 6-well plates were seeded with approximately 200,000 cells per well and incubated at 37°C. After 24 h the culture medium was replaced by fresh medium supplemented with a range of DHA ($>$ 98% pure, Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) concentrations (40, 50, 60, 70, 80, 90, or 100 μM). DHA was added to 10% FCS after which glutamine and geneticin were added (see above). α-Tocopherol (20 μM) ($>$ 96% pure, Sigma) was added standard to the culture medium as a protective agent [23]. Twenty-four hours later cells were harvested, $A\beta_{42}$ content was measured (see $A\beta_{42}$ assay below), and the fatty acid composition of the cell membranes was analyzed. Cell viability after DHA supplementation was measured by a water soluble tetrazolium (WST) (Roche Diagnostics, Almere, The Netherlands) and a lactate dehydrogenase assay (LDH) (Roche) carried out according to the manufacturer's instructions.

For phospholipid analyses, cells were cultured in T175 flasks (Becton Dickinson BV, Alphen aan de Rijn, The Netherlands) and 24 h later culture medium was refreshed and supplemented with 100 μM DHA for another 24 h. Following treatment with trypsin (trypsin EDTA in HBSS, Invitrogen) the number of cells was determined using a Coulter Counter. The cell suspension was centrifuged at 1000 rpm for 5 min, the supernatant removed and cell pellets stored at -20°C for fatty acid analysis.

CMV-luciferase transfection

To control for possible effects of DHA supplementation on promoter activity, additional 24-well plates were cultured with CHO cells. After 24 h the cells were transfected with CMV-luciferase using FuGENE 6 Transfection Reagent (Roche) and incubated with DHA (40, 60, 80, and 100 μM) for another 24 h. Luciferase signal was measured using the Dual-Luciferase Reporter Assay System (Promega Benelux BV, Leiden, The Netherlands). Reduced luciferase activity would be indicative for a reduction of promoter activity as a result of DHA supplementation. Reductions in promoter activity would result in lower levels of AβPP and therefore lower levels of Aβ_{42}.

Aβ_{42} assay

Medium samples (900 μl) taken from supplemented 6-well plates, were loaded on a PD10 desalting column (Amersham Biosciences, Roosendaal, The Netherlands). Samples were concentrated by using a speedvac and dissolved again in 100 μl lysis buffer (62.5 mM Tris-HCl, pH 6.8; 4% SDS and 20% glycerol, according to Prof. Wurtman, Boston, USA). Aβ_{42} analysis was performed using the Signal Select Human Aβ_{42} ELISA assay kit (Biosource BV, Etten-Leur, The Netherlands). The assay was carried out according to the manufacturer's protocol. BSA solution (Omnilabo, Breda, The Netherlands) was used to create a standard curve with a range of 1.5–0.025 mg/ml. Standards and 10-times diluted samples (25 μl) were loaded on a 96-well plate (Becton Dickinson BV) and 200 μl of working reagent was added (1 part BCA Protein Assay Reagent B and 50 parts BCA Protein Assay, both Perbio). After 30 min incubation at 37°C absorbance at 550 nm was measured. Sensitivity of the kit lies between 0 and 1000 pg/mL Aβ_{42} and samples were diluted accordingly to fall within this range. The Aβ_{42} results were corrected for cell numbers and expressed per 1×10^6 cells.

PGE$_2$ assay

PGE$_2$ production was determined by Prostaglandin E$_2$ EIA Kit- Monoclonal (Campro Scientific, Veenendaal, The Netherlands) in medium taken from the 6-well plate. Samples were diluted with EIA buffer (1:3) and processed according to the manufacturer's protocol. The amount of PGE$_2$ (in ng) was corrected for cell numbers. Sensitivity of the kit lies between 2 and 2000 pg/mL PGE2 and samples were diluted accordingly to fall within this range.

COX inhibition

One selective COX-1 inhibitor, SC-560 (VWR International, Roden, The Netherlands), and two selective COX-2 inhibitors, Celebrex (Pharmacia B.V., Woerden, The Netherlands) and curcumin (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands), were used to inhibit the production of PGE$_2$. CHO cells were cultured on a 6-well plate and incubated with one of the COX-inhibitors for 24 h. SC-560 was added in a concentration of 3.75–60.0 nM, Celebrex in a concentration range of 0.037–6.0 μM, and curcumin in 0.0675–27.0 μM. The effects on Aβ_{42} secretion and PGE$_2$ production were determined after 24 h incubation (procedures as described above). In addition, PGE$_2$ levels were measured after 24 h incubation with synthetic PGE$_2$ (0.1, 0.5, 1.5, 3 and 6 pg synthetic PGE$_2$/ml (5Z, 11a, 13E, 15S)-11, 15Dihydroxy-9-oxoprosta-5,13-dienoic acid, Sigma) in the presence or absence of 80 μM DHA (analysis as described above).

Fatty acid analysis

Cell pellets from DHA and COX-inhibitor supplemented cells were dissolved in 250 μl demineralized water. As an internal standard 500 μl 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine was used. Total lipid was extracted from the cells by methanol and chloroform. Subsequently, samples were centrifuged at 3000 rpm for 10 min and the lower phase (chloroform and lipids) was removed. Chloroform was added to the upper phase, samples were centrifuged again at 3000 rpm for 10 min and the lower phase was combined with the first one. The chloroform fractions were dried in a speedvac and the lipid extracts were dissolved in 125 μl chloroform. The phospholipids were separated from total lipid by SPE columns (Bond Elut NH$_2$, Varian).

To determine the fatty acid content of the phospholipids, the phospholipids extract was methylated by adding 0.5 ml 10% BF$_3$. The samples were heated at 100°C for 60 min, and 2 ml hexane and 1 ml sodium hydroxide were added. After vortexing and centrifuging the samples for 5 min at 3000 rpm, the lower phase was vortexed and centrifuged with 2 ml hexane for 5 min at 300 rpm. Top phases of both steps were combined and hexane was evaporated from the samples by means of a speedvac. The fatty acids were dissolved in 125 μl iso-octane and analyzed on a GC-FID with a CP-SIL88 column (50 m $\times$ 0.25 mm id. 0.22 μm film thickness). Data are expressed as amount of fatty acid per 1×10^6 cells.

Phospholipids analysis

The cell pellets were dissolved in 250 μl demineralised water and lipids were extracted using methanol-chloroform. Concentrated lipid extracts (speedvac) were used for the separation and quantification of the phospholipids by HPLC-LSD with a LiChrospher Diol-100 column (250 $\times$ 4.6 mm, 5 μm, Merck). The separation of the phospholipids from the CHO cells was accomplished by a gradient of two mobile phases: 1) hexane: iso-propanole: acetic acid, 2) iso-propanole: water: acetic acid. Both mobile phases were supplied with 0.08% of triethylamine. The gradient changed linearly from 95:5 to 10:90 within 28 min and 1 min after reaching the final ratio, the gradient was changed back to the original composition at a flow rate of 1 ml/min. Peaks were identified by comparison with phospholipid standards (3-sn-phosphatidic acid sodium salt, 3-sn-phosphatidyl-l-serine from bovine brain, 3-sn-phosphatidylethanolamine from bovine brain, L-α-phosphatidylcholine 99% from bovine brain, L-α-phosphatidylinositol ammonium salt from bovine liver and sphingomyelin from bovine brain obtained from Sigma. Data are expressed as amount of phospholipid class per 1×10^6 cells.

Statistical analyses

All data are expressed as means with standard deviations. Data of supplemented cells were compared to cells incubated in control medium. Data were analyzed using ANOVA in SPSS 15.0 (SPSS Inc., Chicago, IL). All $p < 0.05$ were considered significant. If a significant main effect was found with ANOVA, data were subjected to post-hoc testing with Bonferoni correction. Visual inspection of the effect of DHA on Aβ_{42} secretion and the n3/n6 ratio indicated that the two effects were related. The averages of both Aβ_{42} and n3/n6 ratio were plotted for an exploratory correlational analysis and linear regression was performed.

RESULTS

General

Incubation of the CHO cells with the different supplementation conditions did not affect phenotypic appearance of the cells excepting cells incubated with the highest dose of DHA, which showed phenotypic signs of reduced viability. Incubation of CHO cells with different doses of DHA required the addition of α-tocopherol to prevent cell death. To control for possible effects of α-tocopherol on the different read-out parameters, a separate experiment was carried out wherein cells were cultured in plain medium or medium with added α-tocopherol. The results showed that addition of 20 μM α-tocopherol had no effect on PGE_2 production, membrane composition, and on CMV-promoter activity (data not shown). Aβ_{42} secretion was slightly, but not significantly, decreased by α-tocopherol.

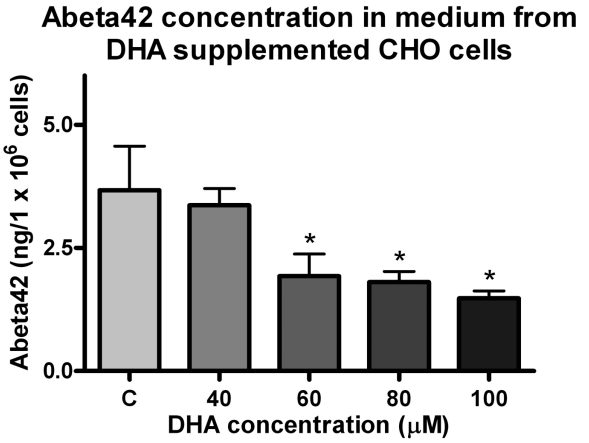

Fig. 1. Dose-dependent decrease in extracellular Aβ_{42} levels after 24 h incubation with DHA (F = 4.2, $p < 0.018$). Data are plotted as ng Aβ_{42} per 1 x 10^6 cells. Asterisk indicates a significant difference compared to C in *post-hoc* analyses.

Aβ_{42} secretion

Incubation of CHO-7PA2 cells with DHA for 24 h resulted in a dose-dependent decrease in Aβ_{42} secretion in the medium (Fig. 1) (F = 4.2, $p < 0.018$). This decrease reached significance at a dosage of 60 μM DHA and higher compared to control, with 100 μM being most effective in lowering Aβ_{42} secretion. These results were confirmed in three separate experiments. The viability of the cells was checked using a WST and LDH test. Cells incubated with doses up to 80 μM DHA showed no changes in viability. Incubation with 100 μM DHA resulted in an 11% decrease of WST levels compared to control and doses up to 80 μM. LDH levels increased by 18% compared to control and doses up to 80 μM. Therefore, the highest dose was not used for further supplementation experiments.

PGE_2 production

Cells supplemented with DHA displayed a 4- to 8-fold decrease in PGE_2 production (F = 8.4, $p = 0.001$)

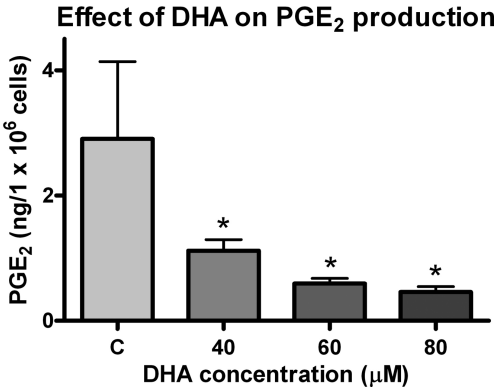

Fig. 2. Extracellular PGE_2 levels after 24 h incubation with DHA. PGE_2 concentrations decreased with increasing DHA concentrations ($F = 12.9$ $p < 0.001$). Data are plotted as ng PGE_2 per 1×10^6 cells. Significant differences compared to C are indicated with *.

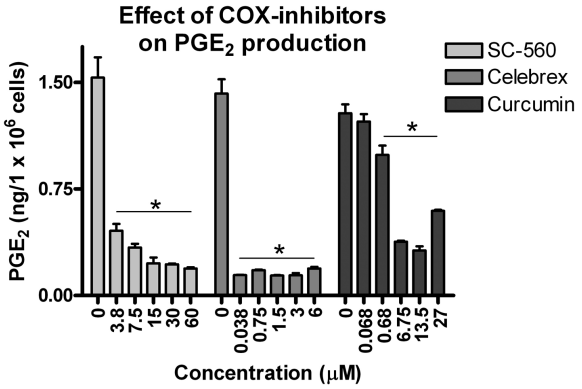

Fig. 3. Extracellular PGE_2 production after 48 h incubation with a selective COX-1 inhibitor (SC-560) or two selective COX-2 inhibitors (Celebrex or curcumin). All three inhibitors show a profound inhibition of PGE_2 production ($F = 63.5$, $p < 0.001$; $F = 153.5$, $p < 0.001$; $F = 82.0$, $p < 0.001$ respectively). Curcumin requires higher dosages to inhibit PGE_2 production, whereas SC-560 and Celebrex show considerable inhibition at the lowest dose used. Data are shown as amount of PGE_2 per 1 x 10^6 cells. Significant differences compared to control dose are indicated with *.

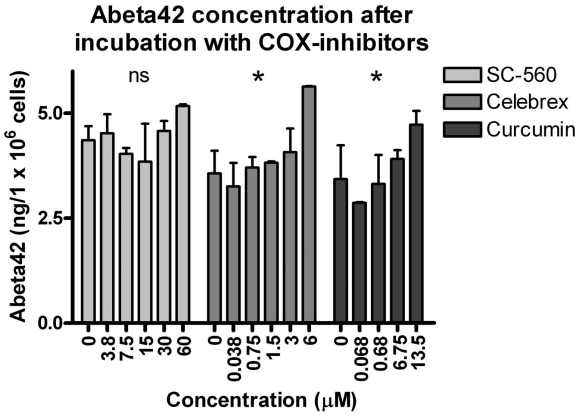

Fig. 4. Secretion of $A\beta_{42}$ after 48 h incubation with a selective COX-1 inhibitor (SC-560) or two selective COX-2 inhibitors (Celebrex or curcumin). ANOVA revealed a dose-depent increase in $A\beta 42$ levels ($F = 5.8$, $p < 0.001$) after incubation with COX-inhibitors. Post-hoc analyses showed that this effect was specific for COX-2 inhibitors (Celebrex and Curcumin) and not for COX-1 inhibitor SC-560 (respectively: $F = 8.8$, $p < 0.001$; $F = 1.1$, $p = 0.46$). Data are plotted as concentration $A\beta_{42}$ per 1×10^6 cells. ns indicates no significant.

(Fig. 2). The effect of DHA supplementation on PGE_2 production was already significant at the lowest dose of DHA used (40 μM) and increased at higher doses. Sensitivity of the kit lies between 2 and 2000 pg/mL PGE_2 and samples were diluted accordingly to fall within this range.

COX inhibition

Supplying the cells with the COX inhibitors SC-560, Celebrex, or curcumin resulted in a reduction of PGE_2 in the culture medium (Fig. 3). SC-560 and Celebrex both completely blocked PGE_2 production, while Curcumin dose-dependently decreased PGE_2 production ($F = 63.5$, $p < 0.001$; $F = 153.5$, $p < 0.001$; $F = 82.0$, $p < 0.001$ respectively). Figure 4 depicts the effects of COX-inhibitors on $A\beta_{42}$ levels. Incubation with COX-inhibitors resulted in a dose-dependent increase in $A\beta_{42}$ levels ($F = 5.8$, $p = 0.001$). Post-hoc analyses revealed that this effect was significant for the COX-2 inhibitors Celebrex and Curcumin ($F = 8.8$, $p < 0.001$) but not for COX-1 inhibition with SC-560 ($F = 1.1$, $p = 0.46$).

Incubation of the cells with synthetic PGE_2 for 24 h increased the level of PGE_2 dose-dependently in the cell medium to respectively 2 and 2.3 fold compared to control at 3 and 6 pg PGE_2/ml. In the presence of 80 μM DHA, PGE_2 levels were significantly lower at all concentrations PGE_2 added, with a 6 fold reduction in the lower ranges and a 2.3 fold reduction in the higher ranges of PGE_2 added (Fig. 5). Cells incubated with DHA and the highest dose of synthetic PGE_2 showed comparable absolute PGE_2 levels compared to cells incubated with the lowest dose of synthetic PGE_2 in the absence of DHA. The membrane n3/n6 ratio was not affected by PGE_2 incubation and equaled 0.95 ± 0.03 (mean $\pm$ S.E.M.) as typically seen in control cells.

Membrane composition

Table 1 presents phospholipid levels of cells incubated for 24 or 48 h with control or DHA supple-

Table 1
Membrane phospholipid levels in cells incubated for 24 and 48 h

	Control 24 h	DHA 24 h	Control 48 h	DHA 48 h
PE	1581.8 ± 22.23	1606.7 ± 26.41	1531.7 ± 0.11	1533.0 ± 1.35
PC	2271.5 ± 62.60	2258.4 ± 59.53	2044.3 ± 1.17	2028.0 ± 5.68
PS	1043.9 ± 7.63	1041.2 ± 5.32	1021.3 ± 0.05	1020.3 ± 0.30
PI	948.2 ± 0.49	948.3 ± 0.62	947.4 ± 0.00	947.4 ± 0.08

Phospholipid levels are expressed in ng per 1×10^6 cells. PE: phosphatidylethanolamine, PC: phosphatidylcholine, PS: phosphatidylserine, PI: phosphatidylinositol.

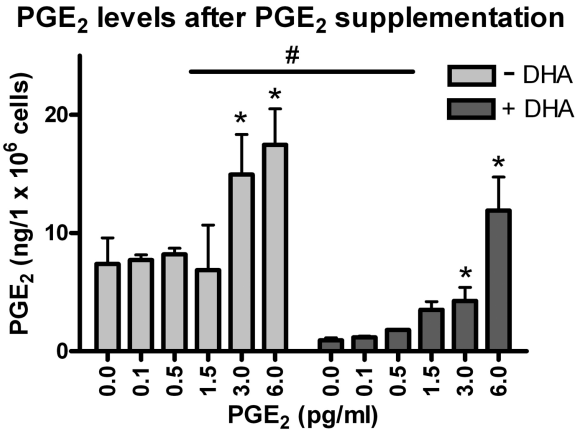

Fig. 5. Concentrations of PGE_2 in the medium after addition of increasing doses of PGE_2alone or in combination with 80 μM DHA for 24 h. Univariate analysis of variance indicated main effects of the factor Dose (having 6 levels) [F = 16.7, $p < 0.001$] and the factor DHA (having 2 levels) [F = 60.4, $p < 0.001$]. No significant interaction between Dose and DHA was found (p = 0.31). Post-hoc comparisons of Doses indicated that: $0 = 0.1 = 0.5 = 1.5 < 3 < 6$ (all $p < 0.015$). # indicates the significant difference between cells incubated without or with DHA. Significant differences compared to the respective control dose are indicated with *.

mented medium. The phospholipid composition of the membranes after 24 h incubation was comparable between DHA and control incubated cells (F = 0.042, $p = 0.838$). To exclude the possibility that time had not been sufficient to affect phospholipid incorporation, cells were also incubated for 48 h. Again no effect of supplementation with DHA on the composition of the membranes phospholipids was found.

Membrane fatty acid composition showed an increase in n-3 PUFAs after 24 h incubation with DHA, in particular the amounts of DHA and EPA were increased (F = 86.3, $p < 0.001$ and F = 45.5, $p < 0.001$ respectively) (Fig. 6). Also, a reduction was found in the total amount of arachidonic acid (AA) as a result of DHA supplementation (F = 5.7, $p < 0.03$) (Fig. 6). Together, these effects resulted in a dose-dependent increase in the n-3/n-6 ratio from 0.9 in control cells to 3.9–5.3 (40 μM and 80 μM respectively) in 24 h DHA supplemented cells (F = 211.5, $p < 0.001$) (Fig. 6). Figure 7 depicts the explorative correlation between the percentage decrease in $A\beta_{42}$ levels compared with control and the percentage increase in the n3/n6 ratio. Regression analysis revealed an $r_s = 0.936$ with a significance of $p = 0.032$.

Promoter activity and cell proliferation

The additional transfection of the CHO-7PA2 cells with a CMV-luciferase construct and incubation with DHA for 24 h resulted in an increased luciferase activity compared to cells incubated without DHA (F = 16.4, $p < 0.001$) (Fig. 8). The effect of DHA supplementation reached significance at a dose of 60 μM.

To control for differences in cell proliferation, cell counts for the different DHA doses were performed. No effect of DHA supplementation was found on cell numbers (data not shown).

DISCUSSION

In humans, consumption of n-3 PUFAs is found to be inversely related to the risk for developing dementia and, in particular, AD [24,25]. Since one of the hallmarks of AD is the accumulation over time of Aβ plaques, in which $A\beta_{42}$ is the predominant species, this study investigated the direct effects of the n-3 PUFA DHA on the secretion of $A\beta_{42}$ in a robust *in vitro* system. The present study clearly shows that DHA supplementation for only 24 h results in a significant dose-dependent reduction of $A\beta_{42}$ in human AβPP transfected CHO-7PA2 cells *in vitro*. Providing these cells with DHA also resulted in a drastic increase in plasma membrane DHA and EPA content and a smaller but significant reduction in AA content which was paralleled by diminished PGE_2 levels. Incubation with COX-inhibitors also resulted in very low PGE_2 levels, although COX-inhibitors did not reduce the rate of Aβ secretion. In fact, incubation with specific COX-2 in-

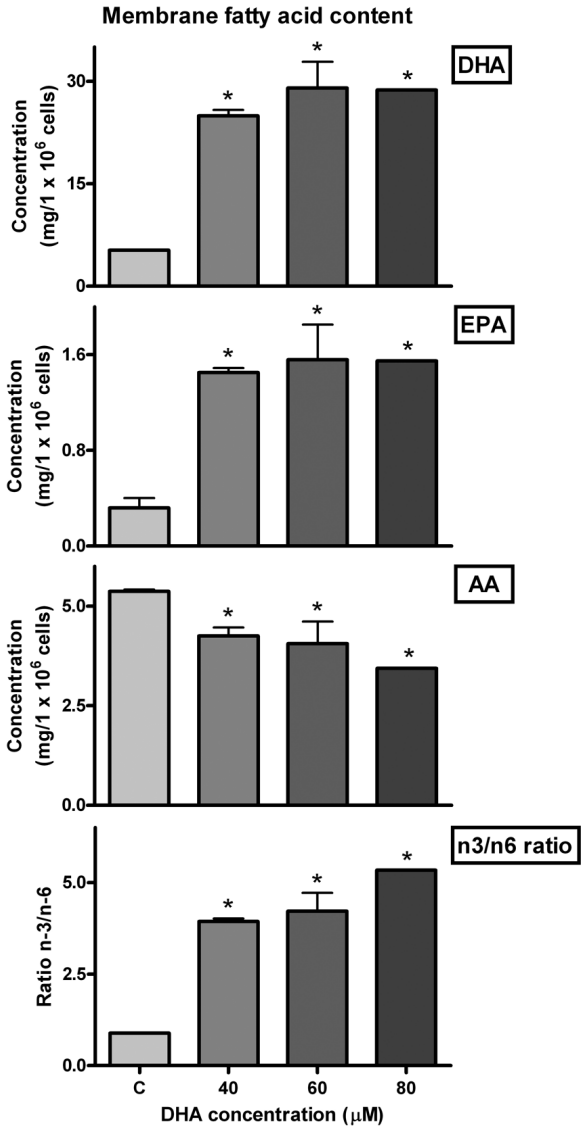

Fig. 6. Fatty acid composition of CHO cells after 24 h incubation with DHA up to 80 μM. Supplementing the cells with DHA resulted in increased levels of DHA and EPA by approximately 500% and decreased levels of AA by more than 30% in the membranes ($F = 42.3$, $p < 0.001$; $F = 20.4$, $p < 0.003$; $F = 7.5$, $p < 0.03$, respectively). DHA in addition increased the total amount of n-3 PUFAs and reduced total amount of n-6 PUFAs resulting in a dose-dependent increase in the n-3/n-6 ratio ($F = 145.2$, $p < 0.001$). Fatty acid data are expressed as the amount in mg per 1 x 10^6 cells. The ratio is calculated as: amount n-3 PUFAs/ amount n-6 PUFAs. Significant differences compared to C are indicated with *.

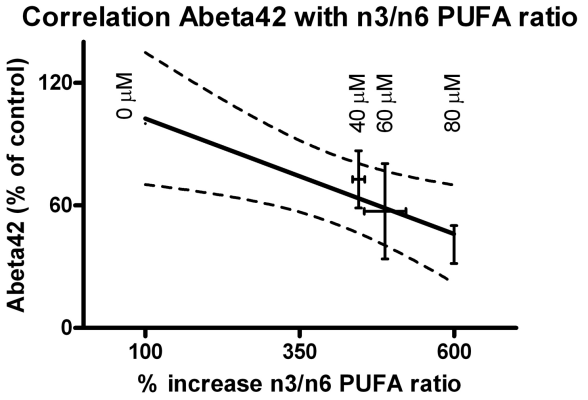

Fig. 7. Explorative correlation between relative Aβ_{42} levels and the percentage increase in membrane n3/n6 PUFA ratio. Plotted are the relative Aβ_{42} levels compared to control as a function of the relative increase in the n3/n6 PUFA ratio. The horizontal and vertical error bars represent the standard errors of the n3/n6 ratio and the standard errors of Aβ_{42}, respectively. A regression line including the 95% confidence interval was plotted ($r_s = 0.936$; $p = 0.032$). Increasing membrane n3/n6 PUFA ratios were associated with reduced Aβ_{42} secretion. The listed concentrations represent the levels of DHA supplementation of the data points.

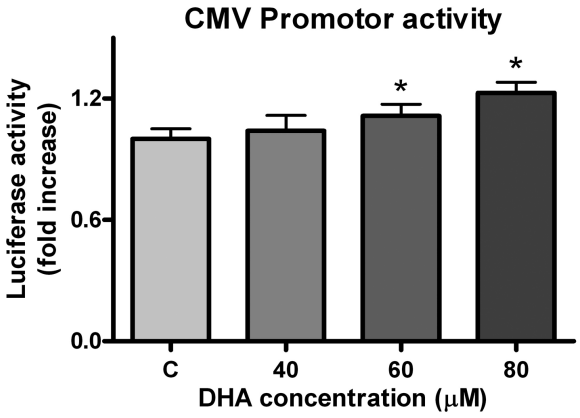

Fig. 8. Luciferase activity of CHO cells incubated with several DHA concentrations. DHA supplementation created a dose-dependent increase in luciferase activity compared to C ($F = 15.0$, $p < 0.001$). Data are plotted as a relative score compared to C.

hibitors induced an increase in Aβ levels. DHA supplementation did not influence cell proliferation and cell viability and survival were not affected with concentrations of DHA lower than 100 μM. The reduction in Aβ_{42} was not the result of lower levels of AβPP as a consequence of reduced promoter activity. Rather, DHA slightly increased promoter activity. The presence of 10% FCS may have contributed in the lowering of Aβ42. Since FCS was present under all testing conditions including the control situation it is unlikely to that FCS has caused the Aβ_{42} reduction. However, a synergistic effect cannot be ruled out.

The effect of DHA was evaluated in the presence of tocopherol. It is known that AD patients, as well as individuals suffering from mild cognitive impairment, show a reduction in plasma antioxidant levels [26–30].

Despite the fact that some studies showed no protective effect of antioxidants [31,32], others demonstrated a reduced risk for developing AD [33–35] in cross-sectional and prospective epidemiological studies. Furthermore, transgenic animals with the AβPP$_{Swe}$ mutation showed reduced oxidative damage and Aβ pathology when provided with antioxidants [36,37]. The exact mechanism, however, through which antioxidants exert their beneficial effects is not clear [38]. Inhibition of key events in inflammatory processes [39] and influencing the phosphorylation state of protein kinase C, a key player in cytokine signaling [40], are suggested as routes of action in addition to the direct antioxidant properties [41,42]. Since the current experimental set-up required the addition of α-tocopherol to prevent oxidation of DHA, it is plausible that the positive results obtained on Aβ secretion are partly due to the combination with antioxidant effects. Indeed, already a small decrease in Aβ secretion, although not significant, was observed in cells supplemented with antioxidants alone. Therefore, it should be kept in mind that the obtained results with DHA are on a background of antioxidants and that a possible interaction cannot be ruled out. As recently suggested [43], the antioxidant basis provided by the tocopherol could have contributed to the magnitude of the effects on amyloidogenic Aβ_{42} production observed following DHA supplementation. Given the several-fold stronger dose-dependent reduction in Aβ by DHA supplementation, the contribution of α-tocopherol is likely to be small.

In our study, DHA supplementation for 24 h resulted in a marked reduction in PGE$_2$ production. Already at a concentration of 40 μM a reduction of 60% was seen, and higher DHA concentrations were even more effective in reducing PGE$_2$. The higher doses of DHA also induced a significant decrease in AA content of the membrane. Since, AA acts as the progenitor of PGE$_2$ [6,44] it seems likely that the decrease in AA levels in the plasma membrane, at least in part, may account for the reduced levels of PGE$_2$. However, such a dramatic suppression of PGE$_2$ formation as a result from a modest reduction in AA content is not supported by evidence from literature. An alternative explanation for this reduction can be found in the competition between AA and EPA for the COX-pathway to form prostaglandins [44–46]. Indeed, the DHA supplementation regime not only affected DHA and AA levels but also increased EPA content of the membrane to 500% of control levels, which is approximately half of the AA membrane content. Supplementing EPA to carcinoma cell lines indeed reduced PGE$_2$ production in a dose-dependent manner while supplementation of comparable doses of AA induced increases in PGE$_2$ levels [47, 48]. Thus, supplementing CHO cells with DHA shifts the balance between AA en EPA in favor of the latter, likely competitively inhibiting the metabolism of AA to PGE$_2$ by COX.

The possible relevance of inflammatory factors in AD is indicated by the elevated levels of COX [49] and PGE$_2$ [14] observed in AD patients. In a series of elegant experiments, Qin et al. [8] showed that Aβ production was increased in transfected CHO-AβPP$_{Swe}$ and H4-AβPP$_{751}$ cells with human COXs compared to non-transfected cells. However, in the current study, the suppression of COX activity by specific COX-1 and COX-2 inhibitors was not paralleled by a reduction of Aβ_{42} formation, despite a marked decrease in PGE$_2$ release. In fact, supplying the cells with the COX-2 inhibitors Celebrex and curcumin promoted Aβ_{42} secretion. Thus, the effects of DHA on Aβ are not dependent on the reduction in PGE$_2$ and seem to be independent of anti-inflammatory properties of DHA. Interestingly, Hoozemans and co-workers showed that addition of AA to the medium, but not of Aβ peptides, increased the release of PGE$_2$ from human microglia and white matter brain tissue independent of COX mRNA transcription [50]. Although, prostaglandins are a contributor to inflammation they are not the sole source of inflammation. It has been shown that AβPP transfection produced stress in cells, not only directly by Aβ production, but also by other metabolites of AβPP such as N-AβPP [51]. Consequently, transfected cells can have activated inflammatory signaling such as NFκB [52], which in turn, could increase COX expression (thus inhibiting COX would not alter Aβ levels). This would suggest that inflammatory processes other than prostaglandins may be contributing to Aβ accumulation.

The molecular mechanisms of action of the n-3 PUFA DHA leading to altered AβPP production or processing and Aβ generation have been subject of several studies [53–56]. These studies show inhibitory effects of DHA on the endogenous or transgenic induced Aβ production in the course of several days [54,56] or weeks [53]. The alterations in membrane DHA content were largely comparable to those observed after 24 h supplementation in our cell cultures. Supplementation of DHA in our cell system not only increased DHA and EPA content of the membranes but also decreased AA content. Inhibition of AA metabolism has been shown to alter the secretion of the N-terminal fragment of AβPP *in vitro* [57]. The stimulatory effect of

AA on AβPP release in another *in vitro* study, however, was comparable to activation of PLA_2, the enzyme that releases AA from cellular stores [58]. This pathway has been implicated in the observed effects of DHA on AβPP processing by stimulating the generation of neuroprotectin D1 from DHA, thereby inducing an anti-apoptotic, neuroprotective gene-expression program that regulates the secretion of Aβ peptides [53]. It has also been suggested that DHA can inhibit the secretion and oligomerization of Aβ directly irrespective of the level of Aβ production [59]. DHA supplementation has also been associated with a reduction in steady-state levels of presenilin 1 thereby directly limiting γ-secretase activity resulting in decreased Aβ production [55]. Finally, the current study did not test and therefore cannot rule out that DHA may have decreased Aβ levels by increasing the levels of sorting and scavenging proteins [56,60–62] or by altering AβPP processing through changed membrane properties as a result of an increased n3/n6 PUFA ratio [56,61] and/or decreased membrane cholesterol content [63,64].

Our current data showing a strong correlation between $A\beta_{42}$ and PUFA ratio, best support the latter pathway and suggests that increasing the n3/n6 ratio decreases Aβ formation and confirms *in vivo* studies with AβPP/PS1 mice showing increased Aβ levels with increased n6/n3 ratios [54]. Future studies should focus on the exact mechanism behind the $A\beta_{42}$ reduction, as well as on effects of combinations of nutrients like nucleotides, B-vitamins or phospholipids, which may enhance the Aβ lowering effect of DHA [65].

In summary, DHA supplementation in the presence of α-tocopherol effectively suppressed $A\beta_{42}$ secretion in human AβPP-transfected CHO-7PA2 cells by 60%. The DHA supplementation resulted in a 500% increase in plasma membrane DHA and EPA content, and a decrease by 30% in AA levels. In addition, DHA supplementation resulted in an 8-fold decrease in PGE_2 production. Cell proliferation and promoter activity were eliminated to be the origin of the observed DHA effects. Moreover, incubation with selective COX inhibitors, that reduced PGE_2 production effectively, did not affect $A\beta_{42}$ secretion. Thus, it is not likely that incubation with DHA reduces $A\beta_{42}$ secretion through PGE_2 related pathways, but rather is a more direct consequence of the increased membrane content of DHA and the altered membrane ratio in n3/n6 PUFAs and/or intracellular events that influence AβPP processing and cleavage directly.

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